STUDIES ON MEGAGAMETOGENESIS IN *CLEMATIS* (RANUNCULACEAE)

by

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INTRODUCTION

Clematis (Ranunculaceae) is a large genus of the temperate regions including about 200 species in both the Old and New Worlds. The plants are commonly called Virgin's Bower or Clematis, and a few species are widely cultivated as ornamentals. They are slightly woody vines or perennial herbs with opposite leaves. The flowers have four (occasionally six) sepals, usually lack petals, and have many stamens and several to many carpels.

In 1898 Bessey described two types of pistils in the Ranunculaceae. In the first type (Tribe Anemoneae) there is a single ovule in each pistil, as in Ranunculus and Myosurus. In a modified form of this type there are two to six rudimentary ovules which develop distal to the functional ovule but never reach maturity. This condition is encountered in Pulsatilla, Anemone, and Clematis. In the second type (Tribe Helleboreae) the pistils have two to many ovules, all or many of which reach maturity, as seen in Delphinium and Caltha. In Clematis the fruit is a one seeded achene.

Vijayaraghavan (1962) found megagametogenesis in C. gauriana Roxb. to be monosporic of the Polygonum Type in the functional ovule and tetrasporic in the sterile ovules.

The purpose of this study is to determine the patterns of female gametophyte development in North American species of Clematis, and also to determine the taxonomic significance (if any) of the embryo sac development.

MATERIALS AND METHODS

The taxa studied (listed by Section according to *Gray's Manual of Botany*) are:

Section	Species	Collection locality and number
Atragene (L.) DC.	*C. orientalis* L.	Clear Creek Co., Colorado *Anderson & Fish 2875*
Viorna Reichenb.	*C. pitcheri* T. & G.	Riley Co., Kansas *Anderson & Fish 2870*
	C. fremontii S. Wats.	Cloud Co., Kansas *Anderson 2629*
Flammula DC.	*C. ligusticifolia* Nutt.	Emery Co., Utah *Anderson & Fish 2884*
	C. virginiana L., f. *missouriensis* (Rybd.) Fern	Wabaunsee Co., Kansas *Fish 6502*
	C. dioscoreifolia Levl. & Vaniot	Cherokee Co., Kansas *Fish 6501*

Voucher specimens are deposited in the herbarium of Kansas State University in Manhattan, Kansas.

A complete series of flower buds from the smallest recognizable through several days after anthesis was collected for each species. In all species, all the buds used came from a single plant, with the exception of *C. fremontii* in which all the buds came from the same population. Between 15 and 20 flower buds were sectioned for each species. This yielded about 12,000 sections mounted on 600 slides.

All materials were fixed and stored in chromic-acetic-formalin (Nawaschin V, Sass, 1958). After a minimum of three days in this fluid the sepals and stamens were removed and the flowers dehydrated in a graded tertiary-butyl alcohol series (Johanson, 1940). The material was then infiltrated with

paraffin in a vacuum oven. The great abundance of uniseriate trichomes on the carpels of C. orientalis and C. virginiana prevented adequate infiltration of the paraffin due to the presence of minute air bubbles trapped in the capillary spaces between the trichomes. This condition was rectified by placing the flowers in a one per cent solution of Aerosol O. T. (a wetting agent) in 70% ethanol, with frequent aspiration, for 48 hours prior to dehydration. The flowers were imbedded in the usual manner and longitudinal sections cut at 9-16 microns with a rotary microtome. The sections were mounted serially on slides with Haupt's adhesive and stained first in Harris' Hematoxylin, then Safranin, finally counterstained with Fast Green, cleared, and mounted in Permount (Sass, 1958).

Five flowers of each species were dissected and the number of floral parts counted; in this manner averages were determined. These values are reported later on in the appropriate places.

OBSERVATIONS

Clematis orientalis L. is a fast growing, slightly woody vine with thin, glaucus, pinnately compound leaves and three-parted or lobed leaflets. The flowers, which are 3-5 cm. across, occur in few-flowered cymes or are occasionally solitary; they have four (rarely six) yellow petaliferous sepals, about 36 stamens and about 136 carpels. The original distribution was from the Himalayan Region to Persia. It was introduced to the United States where it has escaped from cultivation and is now found locally in western North America.

FUNCTIONAL OVULE: The functional ovule in C. orientalis develops at the base of the carpel, and above it four to six sterile ovules are formed (Fig. 1). The growth of the single integument on the lowermost (functional) ovule is accompanied by curvature of the funiculus and the acropetal development of vascular tissue within the ovule (Figs. 2-5). A single subepidermal archesporial cell (Fig. 6) enlarges and functions directly as the megaspore mother cell (Fig. 7), parietal cells are absent. Through meiosis the megaspore mother cell gives rise to a linear tetrad of megaspores (Fig. 8). The three micropylar megaspores degenerate (Figs. 9,10), leaving the chalazal megaspore to function as the embryo sac initial (Fig. 11). The development of the embryo sac is therefore monosporic. The chalazal megaspore nucleus divides to form a two-nucleate embryo sac (Fig. 12); two more mitotic divisions result in the formation of an eight-nucleate embryo sac (Figs. 13,14). The nuclei then migrate to form an organized eight-nucleate megagametophyte (Fig. 15), consisting of a large egg, two smaller synergids, each characterized by a hook-like projection extending toward the micropyle and a basal vacuole, two polar nuclei, and three antipodals in the narrow chalazal end of the embryo sac. The antipodals frequently become binucleate at about the time the polar nuclei fuse to form the secondary nucleus (Fig. 16).

The development of the embryo sac in the functional ovule is as reported by Vijayaraghavan (1962) except that T-shaped tetrads were not observed. The functional ovules of all other species studied develop in a similar manner to C. orientalis.

Variations in the length of the megagametophyte largely reflect the variation in flower size. In each species, five embryo sacs were measured and the average determined. The length of the female gametophyte is as follows: C. orientalis, 118 microns; C. pitcheri, 148 microns; C. fremontii, 125 microns; C. ligusticifolia, 120 microns; and C. virginiana, 125 microns.

STERILE OVULES: In C. orientalis, the sterile ovules are ategmic and lack both vascular tissue and any change in orientation due to curvature. Again, the single subepidermal archesporial cell (Fig. 17) enlarges and functions as the megaspore mother cell (Fig. 18). Meiosis, in this case, is not followed by cytokinesis so the result is a four-nucleate coenomegaspore (Fig. 19). The nuclei migrate to the center of the cell and the cytoplasm becomes highly vacuolated at each end (Figs. 20,21). The nuclei then fuse in pairs (Fig. 22) either longitudinally or laterally, giving rise to a secondary two-nucleate embryo sac with diploid nuclei (Fig. 23). The development of the embryo sac is therefore tetrasporic. These nuclei divide by mitosis (Fig. 24) to form a secondary four-nucleate embryo sac (Fig. 25). Following this, some or all of the nuclei form cell walls and become oriented in one of the following patterns (in order of frequency): (1) an organized four-nucleate embryo sac with an egg, two polar nuclei, and one antipodal (Fig. 28), (2) all four nuclei in a linear row of cells (Figs. 26,27), or (3) a four-nucleate embryo sac with an egg, one polar nucleus, and two antipodals (Fig. 29). Very rarely an eight-nucleate embryo sac is formed (Fig. 30); apparently

it is formed by a division of the secondary four-nucleate embryo sac before it becomes organized. The eight-nucleate embryo sac is of further interest in that the egg apparatus is in the chalazal end. These cells are persistent until after fertilization of the functional embryo sac, then the nuclei degenerate.

Discussion of the following species will be limited to the development of their sterile ovules. In all species studied evidence of fertilization in the sterile ovules was lacking and seeds were never formed.

Clematis pitcheri T. & G. is a climbing woody vine with pinnately compound leaves, each having three to nine leaflets. The flowers, which are 2½-3 cm. long and are borne in few-flowered cymes, have a campanulate calyx of four purple sepals with recurved tips, about 110 stamens and an average of 77 carpels. This species ranges from western Indiana to Iowa and southeast Nebraska and south to western Tennessee, Arkansas, and Texas.

In C. pitcheri the two or three sterile ovules begin development somewhat later than those of C. orientalis, and they also develop at a slower rate (Table 1). The most advanced stage observed was the secondary two-nucleate embryo sac, seen in a carpel in which the functional ovule had an organized eight-nucleate megagametophyte. At this time the nuclei degenerate and the resulting embryo sac in the sterile ovule is empty before the functional embryo sac is mature.

Clematis fremontii S. Wats. is a small herbaceous subshrubby plant with thick, crowded, simple leaves. The flowers, which are 2½-3 cm. long, are solitary with a campanulate calyx of four

purple sepals with recurved tips, about 80 stamens and an average of 42 carpels. This species is restricted to northcentral Kansas and eastcentral Missouri where it is represented by C. f. var. riehlii Erickson.

The development of the two or three sterile ovules in C. fremontii begins at about the same time as those of C. pitcheri (Table 1). However, the developmental rate is faster and a secondary two-nucleate embryo sac is present in the sterile ovule at the same time the functional ovule has a four-nucleate embryo sac. At this time the ovary wall enlarges and closes the locule above the functional ovule, thus crushing the sterile ovules.

An interesting anomaly found in C. fremontii was that in one functional ovule there were two megagametophytes. One was eight-nucleate and the other was seven-nucleate due to the fusion of the polar nuclei. In both, the antipodals were uninucleate and the length of the embryo sac was 159 microns.

Clematis ligusticifolia Nutt. is a woody climber with pinnately compound leaves, each having five leaflets. The dioecious plants have about 6-20 white flowers, each 6-12 mm long, borne in corymbiform panicles. Each pistilate flower has four sepals, several staminodia, and an average of 62 carpels. The staminate flowers are the same size and have four sepals and an average of 30 stamens. This species ranges from Manitoba to British Columbia south to western Missouri, Kansas, New Mexico, Arizona, and California.

Carpels of C. ligusticifolia have one or two sterile ovules. Archesporial cells are not recognizable until much later in

development and very few of these function as megaspore mother cells. In fact, meiosis had reached completion in only one sterile ovule. Apparently further development is arrested as no functional megaspores were found.

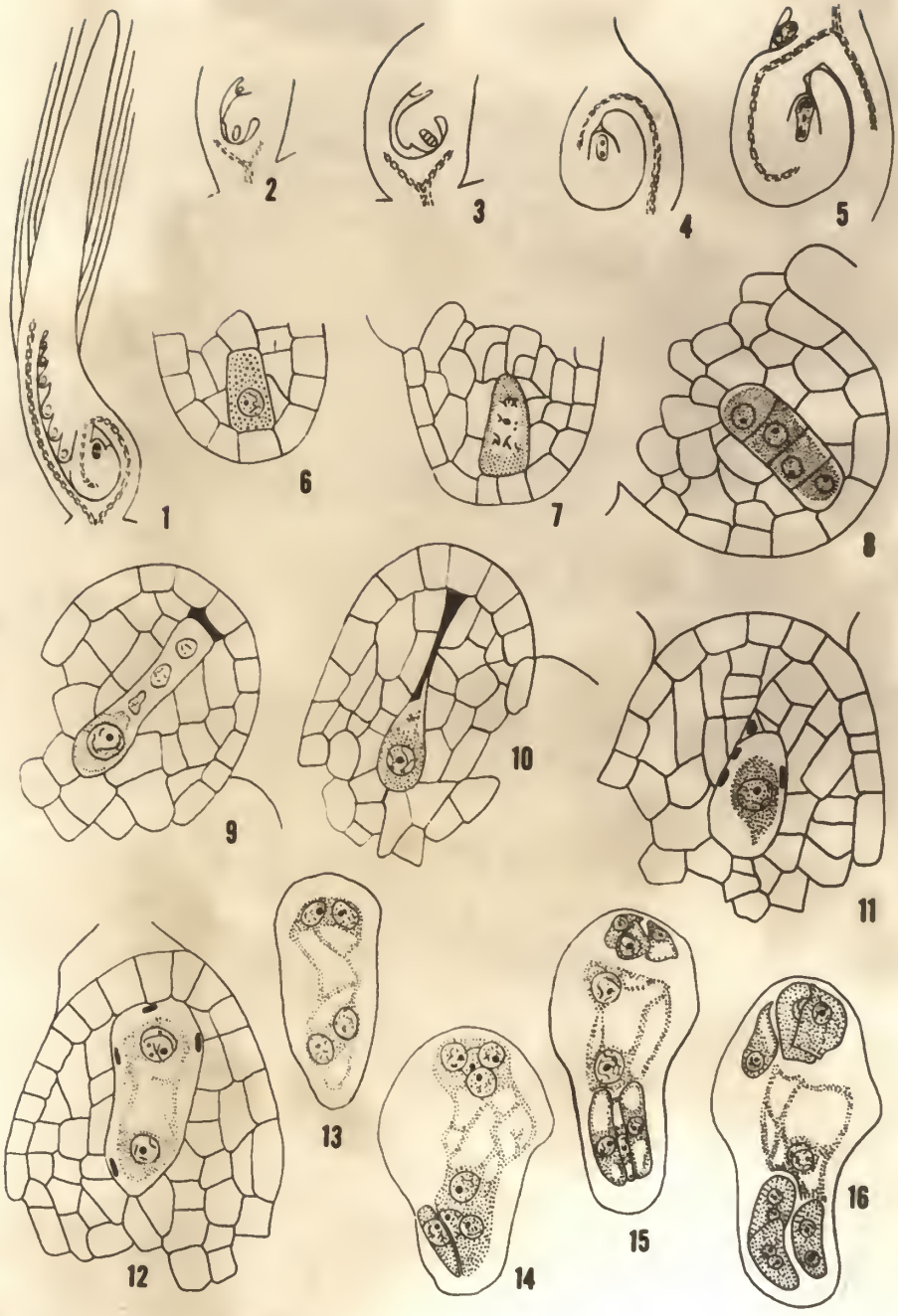
Clematis virginiana L. f. missouriensis (Rybd.) Fern is a woody climber with three-foliate leaves, densely pilose beneath. About 20-35 pistilate flowers, each 6-12 mm long, and having four white sepals, several staminodia, and an average of 60 carpels, form a corymbiform panicle. The staminate flowers are the same size and each has four sepals and an average of 35 stamens. This species is found from Quebec to Manitoba south to Georgia, Alabama, Louisiana, and eastern Kansas.

Sterile ovules are absent in C. virginiana. The locule does not extend above the functional ovule and thus there is no room for sterile ovules to develop.

Clematis dioscoreifolia Levl. & Vaniot is a glabrous, woody high climber. Each leaf is divided into five round-ovate leaflets, each with rounded tips and chordate bases. About 7-12 flowers form a corymbiform panicle. Each flower, which is 1-2 cm. long, has four narrow white sepals, about 35 stamens, and an average of six carpels. This species ranges from Massachusetts to Virginia; spreading locally from cultivation.

The flowers of C. dioscoreifolia were heavily infested with insect larvae; therefore very few satisfactory sections were obtained. Each carpel has three or four sterile ovules, and these show development to at least the organized four-nucleate stage similar in appearance to those seen in C. orientalis.

Figs. 1-16. Development of functional ovule in C. orientalis, receptacle toward bottom of page in each drawing. Fig. 1. Longisection of carpel showing one fertile ovule and six sterile ovules. X30. Figs. 2-5. Longisections of fertile ovules showing progressive stages in development and curvature. X50. Fig. 6. Archesporial cell. Fig. 7. Meiosis in megaspore mother cell. Fig. 8. Linear tetrad of megaspores. Figs. 9,10. Stages of degeneration of the three micropylar megaspores. Fig. 11. Functional megaspore. Fig. 12. Two-nucleate embryo sac. Fig. 13. Four-nucleate embryo sac. Fig. 14. Unorganized eight-nucleate embryo sac. Figs. 15,16. Organized female gametophytes, the latter showing binucleate antipodals. Figs. 6-16, X480.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Figs. 17-30. Developmental sequence in sterile ovules in C. orientalis. Fig. 17. Archesporial cell. Fig. 18. Megaspore mother cell. Fig. 19. Four-nucleate coenomegaspore. Fig. 20, 21. Four-nucleate coenomegaspore showing nuclear migration and development of vacuoles. Fig. 22. Lateral fusion of pairs of megaspores. Fig. 23. Secondary two-nucleate embryo sac. Fig. 24. Division of two-nucleate embryo sac; nuclei in anaphase. Fig. 25. Secondary four-nucleate embryo sac. Figs. 26,27. Organized embryo sacs; all four nuclei have organized into cells. Fig. 28. Same, with an egg, two polar nuclei, and one antipodal. Fig. 29. Same, with an egg, a polar nucleus and two antipodals. Fig. 30. Eight-nucleate embryo sac with an egg, two synergids, two polar nuclei, and three antipodals; note that the egg apparatus is at chalazal end of embryo sac. All, X540.

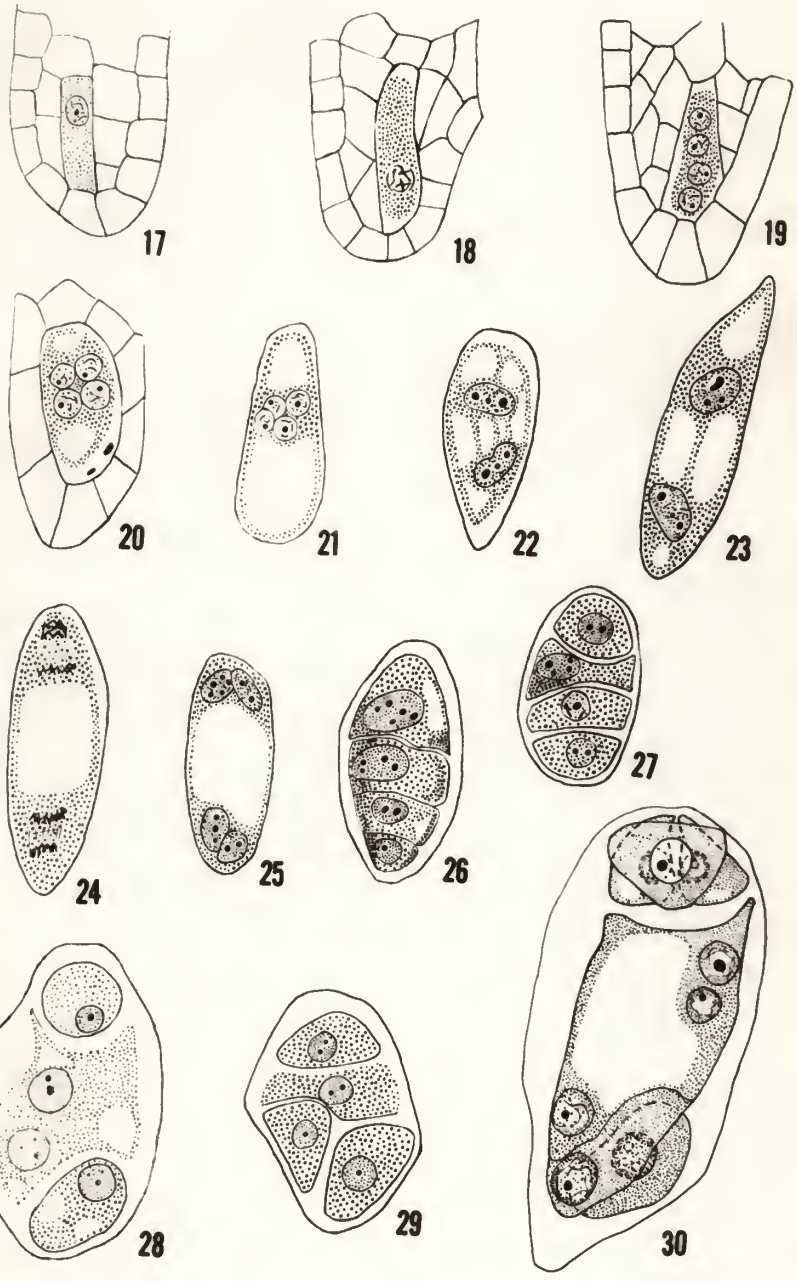
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Table 1.

Correlation of Successive Stages of Development
in Functional and Sterile Ovules.

Functional ovule in all species	Sterile Ovules			
	C. orientalis	C. pitcheri	C. fremontii	C. ligusticifolia
Archesporial cell	Archesporial cell			
Megaspore mother cell	Megaspore mother cell	Archesporial cell	Archesporial cell	
Tetrad	4-nucleate coenomegaspore	Megaspore mother cell	Megaspore mother cell	
Functional megaspore	as in Fig. 21	4-nucleate coenomegaspore	4-nucleate coenomegaspore	
2-nucleate embryo sac	Fusion and sec. 2N e-sac		as in Fig. 20	
4-nucleate embryo sac	Division to sec. 4N e-sac	as in Fig. 20	Fusion and sec. 2N e-sac	Archesporial cell
8-nucleate embryo sac	Unorganized sec. 4N e-sac	Fusion of megaspores		
Organized gametophyte	Organized 4 & 8N embryo sacs	Secondary 2N embryo sac		4-nucleate coenomegaspore

DISCUSSION

Bessey (1898) stated that it was unlikely that the strictly uniovulate carpels were derived from the multiovulate carpels of the Ranunculaceae. Instead he proposed "that some plant of the Ranunculus or Myosurus type after the development of its first ovule varied so as to develop in the space above the ovule one or more accessory ovules which were unable to reach maturity." Nevertheless, Maheshwari (1963, p. 62) stated that the presence of such ovules marks an intermediate stage in a reduction series from a multiovulate to a uniovulate carpel. This is in keeping with C. E. Bessey's thinking as reflected in his Dicta (1915).

All the Clematis species studied have a basal functional ovule with several sterile ovules above it, with the exception of C. virginiana L. f. missouriensis (Rybd.) Fern in which sterile ovules are absent.

The unitegmic functional ovule is borne on a curved funicle with a vascular supply. In all species studied, the embryo sac development is monosporic, of the Polygonum type, resulting in the formation of an eight-nucleate megagametophyte in which the antipodals are commonly binucleate. T-shaped tetrads as reported by Vijayaraghavan (1962), were not observed.

In the distal (non-functional) ovules there is no vascular supply, micropyle, or integument. The differences between species are manifested in the extent of development of the sterile ovules and by their absence in the case of C. virginiana. Development is tetrasporic and in C. orientalis results in the formation of both four and eight-nucleate embryo sacs which have

diploid nuclei due to the fusion in pairs of the megaspore nuclei. Development of a haploid embryo sac as reported by Vijayaraghavan (1962) was not observed. The egg apparatus of the eight-nucleate megagametophyte is in the chalazal end of the embryo sac. This suggests that there is marked variation in the polarity of the sterile embryo sac as Vijayaraghavan (1962) described the egg as being in the micropylar end of the embryo sac.

The data obtained in this study of the embryo sac development illustrates a linear sequence in development, from carpels with abundant sterile ovules, all or nearly all of which develop organized embryo sacs, through carpels which show a reduction in sterile ovule number and degree of development within them, to carpels in which sterile ovules are absent (Table 1). This sequence can be (and has been) read in both directions; E. A. Bessey thought of an increase in ovule number as an advanced characteristic but C. E. Bessey looked upon a reduction in ovule number as an advancing evolutionary trend.

In the determination of phylogenetic trends it is best to correlate several characteristics at the same time. For this reason data on the development of the embryo sac will be coupled with other morphological features related to the flowers and inflorescences.

In this manner it can be seen that C. orientalis is possibly the least specialized of the species studied. Some primitive characteristics present in this species, as compared with those of the other species, are: solitary flowers or few-flowered

inflorescences, large flowers, abundance of carpels, and relative abundance of sterile ovules nearly all of which produce an organized gametophyte (Table 2).

On the basis of embryo sac development in the sterile ovules, C. pitcheri is certainly further removed from a multiovulate ancestor than is C. orientalis. Flowers of C. pitcheri have a reduction in size, carpel number, sterile ovule number and relative amount of development within the sterile ovules. The flowers have an increase in the number of stamens.

Clematis fremontii is quite similar to C. pitcheri in both gross morphology and embryo sac development. Flowers of C. fremontii have a reduction in both the stamen and carpel number, as compared with C. pitcheri. The presence of solitary flowers, simple leaves, and herbaceous habit tend to indicate that C. fremontii arose through reduction of an ancestor similar to C. pitcheri.

Clematis ligusticifolia and C. virginiana are very similar in both gross morphology and embryo sac development. They both seem to be highly derived in that they are dioecious, have many-flowered inflorescences, small flowers, and have a reduction in carpel number and sterile ovule number. Carpels of C. ligusticifolia have one (rarely two) sterile ovule and development within it rarely reaches the four-nucleate coenomegaspore. It has been previously stated that sterile ovules are absent in C. virginiana.

While Gray places C. dioscoreifolia in the section Flammula along with C. ligusticifolia and C. virginiana, on the basis of

its gross morphology, my observations indicate that it has been misplaced. Carpels of C. dioscoreifolia have three or four sterile ovules which develop to at least the organized four-nucleate stage similar to that seen in C. orientalis, although the number of carpels is greatly reduced. Here is a case of extreme reduction in the number of carpels while the carpels themselves are relatively primitive. My observations tend to indicate that C. dioscoreifolia most likely arose through the reduction of a plant similar to C. orientalis where sterile ovules are frequent and reach a high level of development, rather than by reduction of a plant like C. ligusticifolia or C. virginiana in which sterile ovules show very little development or are absent.

With the exception of the number of stamens in C. pitcheri and C. fremontii, all the floral characteristics can be read as a reduction series while the number of flowers in a single inflorescence increases. The increase in stamen number in the section Viorna indicates that phylogenetic trends are not always manifested to the same degree in all parts of the plant. These observations strongly support Bessey's view that reduction in flower parts is an advanced characteristic.

While the data is arranged in a linear series for the purpose of clarity, it is not intended to show the origins of the sections. It seems most probable that the sections all arose from one obscure ancestor and then evolved in slightly different directions, as it is unlikely that the section Viorna arose directly from the section Atragene, and even less likely that

the section Flammula arose directly from either the section Viorna or the section Atragene.

Table 2.

Selected Morphological Characteristics of Species of *Clematis*.

Characteristic	Plant Species					
	C. orientalis	*C. pitcheri*	*C. fremontii*	*C. dioscoreifolia*	*C. ligusticifolia*	*C. virginiana*
Number of flowers per inflorescence	1-3	2-3	1	7-12	6-20	20-35
Flower size	3-5 cm	2½-3 cm	2½-3 cm	1-2 cm	6-12 mm	6-12 mm
Number of stamens	36	110	80	35	30	35
Number of carpels	136	77	42	6	62	60
Number of sterile ovules	4-6	2-3	2-3	3-4	1 (2)	0

LITERATURE CITED

Bessey, C. E. 1915. Phylogenetic taxonomy of flowering plants. Ann. Mo. Bot. Gard. 2: 109-164.

Bessey, E. A. 1898. The comparative morphology of the pistils of the Ranunculaceae, Alismaceae, and Rosaceae. Bot. Gaz. 26: 297-313.

Fernald, M. E. 1950. Gray's manual of botany. Eighth Edition. American Book Co. New York, N. Y.

Johanson, D. A. 1940. Plant microtechnique. McGraw-Hill. New York, N. Y.

Maheshwari, P. 1963. Recent advances in the embryology of angiosperms. Internat'l Soc. Plant Morph. Delhi, India.

Sass, J. E. 1958. Botanical microtechnique. Third Edition. The Iowa State University Press. Ames, Iowa.

Vijayaraghavan, M. R. 1962. Studies in the family Ranunculaceae. II. The female gametophyte of Clematis gauriana Roxb. Phytomorphology. 12: 45-49.

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The embryo sac development in *Clematis orientalis*, *C. pitcheri*, *C. fremontii*, *C. ligusticifolia*, *C. virginiana* f. *missouriensis*, and *C. dioscoreifolia* is described. The development of the functional ovule is monosporic and development of the sterile ovules is tetrasporic. The number of sterile ovules and amount of development within them is correlated with other morphologic characteristics and the relationships among these *Clematis* species are discussed.